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Characterization of the Humoral Immune Response in Heparin-Induced Thrombocytopenia

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Recent reports indicate that antibodies associated with heparin-induced thrombocytopenia and thrombosis (HITP) are specific for complexes formed between heparin and the heparin-binding, platelet alpha granule protein, platelet factor 4 (PF4). As with other disorders mediated by immune complexes (IC), the characteristics of the involved immunoglobulins could affect the ability of IC to cause symptoms. We therefore studied the class, subclass, and potency of antibodies specific for heparin:PF4 complexes formed by two groups of patients: one with severe thrombocytopenia, with or without thrombosis, and a positive serotonin release assay (SRA) (Group 1) and another with mild or absent thrombocytopenia, absence of thrombosis, and a negative SRA despite having formed antibodies reactive with heparin:PF4 complexes (Group 2).

IgG antibodies were more common in the Group 1 patients (100%) than in Group 2 (46%), whereas IgM antibodies were more common in Group 2 (81%) than in Group 1 (42%) ($P = 0.009$). About half of each group formed IgA antibodies. In each group, the IgG antibodies were predominantly IgG1 (82%); 42% were IgG3. Only one IgG2 antibody was identified in a total of 52 antibody formers. Antibodies of the IgG class were consistently of higher titer in Group 1 patients than in Group 2 patients ($P < 0.001$).

Recent reports suggest that the H131 form of the FcγRII receptor, which binds preferentially to IgG2 Fc, is found with greater than expected frequency in patients with HITP. Identification of only one IgG2 antibody among 38 antibodies of the IgG class argues against a unique role for antibodies of this subclass in the pathogenesis of HITP. The finding that titers of antibodies in Group 1 patients were a significantly higher titer than in Group 2 patients suggests that development of the full-blown HITP syndrome may require the formation of antibodies of unusually high titer. Am. J. Hematol. 54:196–201

Key words: heparin-induced thrombocytopenia/thrombosis; heparin; PF4; FcγRII receptor; PF4; heparin ELISA; serotonin release assay; isotyping

INTRODUCTION

Heparin-induced thrombocytopenia/thrombosis (HITP) is a common and often serious complication of heparin therapy [1–3]. About 5–10% of patients treated with heparin for 5–7 days develop thrombocytopenia, and roughly 10% of these experience thrombotic complications [1–3]. For some time, it has been known that HITP is associated with the formation of antibodies that activate platelets in the presence of heparin. Because this activation could be blocked by antibodies specific for the platelet FcγRII receptor [4], it was suspected that immune complexes might play a role in platelet destruction. However, direct interaction of the antibodies with heparin to form complexes could not consistently be demonstrated [5]. Recent observations from several laboratories have shown that antibodies associated with HITP are

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specific not for heparin, but for complexes of heparin and platelet factor 4, a basic protein normally found in platelet alpha granules [6–10].

Nearly all patients with classical HITP have immunoglobulins in their serum that recognize heparin:PF4 complexes [6–10]. These can be of the IgG, IgM, or IgA class [7,10], but the frequency with which antibodies of the different classes are formed, their relationship to the immunization process and to clinical disease, and the importance of antibody potency (titer) to symptomatology have not yet been defined. Recent reports have shown that 10–30% of patients given heparin for treatment or prevention of thrombosis [10,11] and about 60% of those anticoagulated during and after open heart surgery [12] form antibodies reactive with heparin:PF4 without manifesting thrombocytopenia or thrombosis. Thus, factors other than the presence of antibodies per se, appear to be important in the pathogenesis of HITP. Two recent reports have indicated that HITP is more likely to occur in patients whose platelets express an allele of the FcγRII receptor containing histidine at position 131 in the peptide chain [13,14]. This is of interest because antibodies of the IgG2 subclass bind preferentially to this form of FcγRII [15].

It is apparent that more information about the Ig classes, subclasses, and potency of antibodies associated with HITP and their relationship to clinical symptoms is needed. To address these questions, we studied antibodies formed in response to heparin by two groups of patients. The first group had classical HITP with or without associated thrombosis. The second was given heparin in conjunction with open heart surgery and experienced no hematologic changes or thromboses that could be attributed to heparin exposure.

MATERIALS AND METHODS

Patient Populations

Group 1 consisted of 26 patients (10 males and 16 females ranging in age from 46 to 82 years) who developed thrombocytopenia (platelets < 100,000/μL) while receiving unfractionated porcine heparin for the prevention or treatment of thrombosis. Seven of these individuals experienced new or additional thrombotic symptoms (deep vein thrombosis and/or pulmonary embolism) and four developed disseminated intravascular coagulation, which led to a fatal outcome in two instances. In each case, the diagnosis was established by a positive serotonin release test [16] and, subsequently, by the demonstration of antibodies reactive with heparin:PF4 complexes (see below). The 26 patients in Group 2 (19 males and 7 females ranging in age from 32 to 75 years) were treated with heparin during and for 1–3 days after open heart surgery. They formed antibodies reactive with heparin:PF4 complexes within 6 days of surgery, but did not exhibit a positive serotonin release test or experience thrombosis or DIC. Eleven of the Group 2 patients (42%) developed moderate thrombocytopenia postoperatively, as would be expected in patients undergoing surgery with cardiac bypass [17]. The Group 2 patients were part of a cohort studied to determine the frequency with which antibodies reactive with heparin:PF4 complexes develop in patients given heparin during open heart surgery [12]. The 26 patients whose antibodies were analyzed in greater detail were selected on the basis of the availability of plasma or serum in sufficient quantity to determine antibody class and subclass. Characteristics of the two groups of patients are summarized in Table I. The study was approved by a Human Research Review Committee and all patients gave informed consent.

TABLE I. Characteristics of the Patient Populations Studied*

Patient group	No. of patients	Symptoms	Heparin:PF4 ELISA	SRA
1	26	Severe TP ± TH	Positive	Positive
2	26	Absent	Positive	Negative

*SRA = serotonin release assay; TP = thrombocytopenia; TH = thrombosis.

Heparin Exposure

Patients from Groups 1 and 2 were comparable in respect to age, indication for heparin therapy, and duration of treatment. All were exposed to unfractionated porcine heparin (UFH) administered by intravenous drip for 1–3 days. In both groups the total dose of heparin received ranged from 25,000 to 450,000 U (median 50,000 U).

Reagents

Heparin sodium derived from porcine intestinal mucosa was obtained from Elkins-Sinn, ESI Pharmaceuticals (Cherry Hill, NJ). Human PF4 was purified as previously described [5]. The affinity-purified IgG human subclasses (IgG1-kappa, IgG2-kappa, IgG3-kappa, and IgG4-kappa) from human myeloma proteins were obtained from Sigma Immuno Chemicals (St. Louis, MO), affinity purified human IgA were obtained from Jackson Immune Research (Westgrove, PA), and affinity purified human IgM from Organon Teknika Cappel Scientific Division (Malverne, PA). Affinity-purified, alkaline phosphatase-labeled mouse anti-human IgG subclasses (heavy chain specific anti-IgG1, anti-IgG2, anti-IgG3, and anti-IgG4), anti-human IgA and IgM, and p-nitrophenylphosphate (PNPP), were purchased from Zymed Labs, Inc. (San Francisco, CA). Ecteola cellulose (epichlorohydrin triethanolamine cellulose), and Tween-20 were purchased from Sigma Chemical Co. Fetal bovine serum (FBS) was obtained from Hyclone Labs (Logan, UT).

Patient Samples

Samples consisted of serum or EDTA platelet-poor plasma. Each sample was absorbed with Ecteola cellulose as previously described [7] to remove residual heparin. Samples were stored at −80°C before being studied.

Heparin:PF4 ELISA

The heparin:PF4 ELISA was performed as previously described [7]. In brief, heparin:PF4 complexes were produced by incubating PF4 (10 μg/mL) with heparin (0.4 U/mL). In previous studies, we showed that heparin and PF4, combined at this ratio, form complexes optimal for detection of antibodies associated with HITP [7]. Fifty microliter aliquots of the heparin:PF4 mixture were incubated overnight at 4°C in the wells of a polystyrene microtiter plate. Fifty microliter aliquots of PF4 alone at a concentration of 10 μg/mL were added to control wells. The plates were washed with 0.02 M phosphate-buffered sodium chloride (PBS) pH 7.2, containing 0.05% Tween 20 (PBS-Tw) and were then blocked at room temperature with PBS-Tw containing 20% fetal bovine serum (PBS-Tw-FBS). Fifty microliters of patient serum or plasma at various dilutions was added to each well and incubated for 60 min at room temperature. After washing with PBS-Tw, 50 μL of alkaline phosphatase-labeled mouse monoclonal anti-human Ig reagent specific for IgA, IgM, or IgG subclasses 1, 2, 3, or 4 was added, followed by incubation for 60 min at room temperature, washing four times, and incubating with p-nitrophenylphosphate (PNPP) substrate for 90 min. Reactions were read at 405 nm using 650 nm as reference. A positive reaction was defined as one whose OD exceeded the mean OD plus 3 SD obtained in wells containing PF4 alone.

Statistical Analysis

Statistical analysis was performed with the Primer of Biostatistics (Stanton A. Glantz, McGraw-Hill, Inc.,) software package. The Chi square test and the Mann-Whitney rank sum test were used in making comparisons between groups. A P value of 0.05 or less was considered to be statistically significant.

RESULTS

Validation of the Isotype and Subclass-Specific Probes

The probes used to identify the class and subclass of immunoglobulins reactive with heparin:PF4 complexes in ELISA have been previously characterized for monospecificity [18–20]. We further validated these probes by testing their reaction against affinity-purified human immunoglobulins of known class and subclass (see Reagents). Fifty microliters (100 ng) of the purified immunoglobulins diluted in 0.05 M sodium carbonate buffer (pH 9.7) were added in triplicate to wells of a polystyrene microtiter plate (Easywash, Corning, NY) and incubated overnight at 4°C. The plates were washed three times with 0.02 M PBS-Tw and blocked for 30 min at room temperature with 0.02 M PBS-Tw-FBS 20%. Fifty microliters of alkaline phosphatase-labeled mouse monoclonal antibody-specific for individual Ig classes or IgG subclasses, diluted 1:250 in PBS-Tw-FBS 10%, were added to each well. In preliminary studies, this amount of the alkaline phosphatase-labeled probes was found to be saturating. After 60 min incubation at room temperature, the bound, labeled probe was detected with PNPP substrate as described above. As shown in Figure 1, each of the probes reacted only with immunoglobulin of the Ig class or IgG subclass for which it was specific.

Immunoglobulin Class of HITP Antibodies

Classes of immunoglobulins reactive with heparin:PF4 complexes formed by patients in Groups 1 and 2 are summarized in Table II. Each of the 26 Group 1 patients (100%) had IgG antibodies. Eleven (42%) also had IgM antibodies. Accompanying IgA antibodies were found in 15 (58%). In contrast, IgG antibodies were detected in only 12 (46%) of the 26 Group 2 patients, while 21 (81%) had IgM and 12 (46%) had IgA antibodies. The increased frequencies of IgG antibodies in Group 1 (100 vs. 46%) and of IgM antibodies in Group 2 (81 vs. 42%) were statistically significant ($\chi^2 = 6.772$, $P = 0.009$).

Subclass of IgG Antibodies

As shown in Table III, 25 of the 26 IgG antibodies in the Group 1 patients were of subclass 1, 3, or 1 + 3. The dominant antibody subclass was IgG1 (24 of 26, 92%). Only one relatively weak (titer 1:50) IgG2 antibody was identified in combination with a much stronger (titer > 1:500) IgG3 antibody. The 12 IgG antibodies identified in the Group 2 patients were exclusively of the IgG1, 3, or 1 + 3 subclasses.

Antibody Potency

The titers of antibodies of all three immunoglobulin classes were generally higher in the Group 1 (SRA positive) patients than in the Group 2 (SRA negative) patients, in whom only two of the 26 antibodies (one IgG and one IgA) titered more than 1:100 (Fig. 2). However, only the difference in IgG titers was statistically significant ($P < 0.001$). The titers of IgG1 and IgG3 antibodies were roughly comparable (data not shown). There was no consistent relationship between the titers of antibodies of different classes and subclasses formed by the same patient.

DISCUSSION

The recent finding that patients with immunologically mediated (Type II) heparin-induced thrombocytopenia

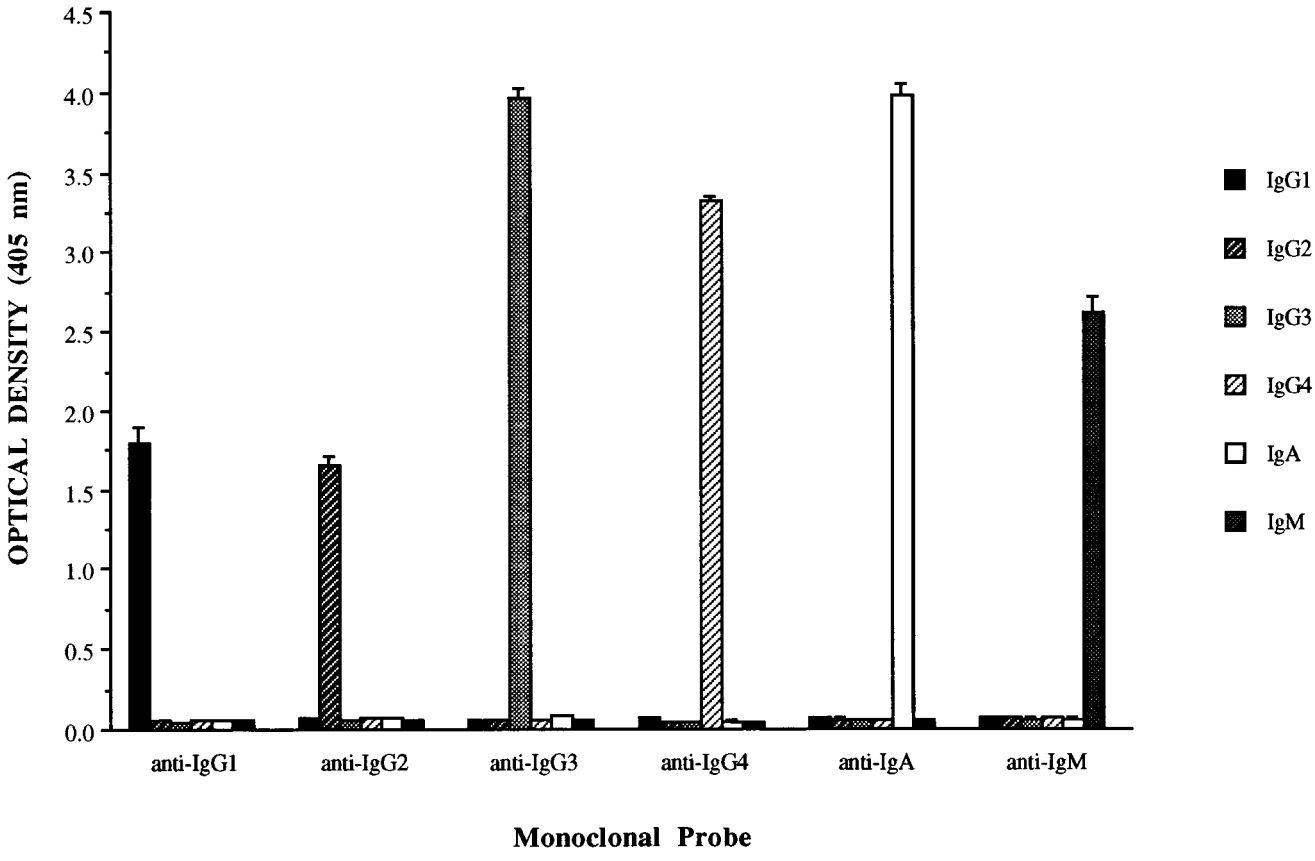

Fig. 1. Reactions of alkaline phosphatase-labeled IgG monoclonal probes with immobilized human monoclonal immunoglobulins of known class and subclass (mean of triplicate determinations + 1 SD). Each probe reacted only with immunoglobulin of the class or subclass for which specificity was claimed by the supplier.

TABLE II. Immunoglobulins Reactive With Heparin:PF4 Complexes Detected in Plasma From Patients in Groups 1 and 2

Immunoglobulins detected by	Group 1 (N = 26)	Group 21 (N = 26)
IgG only	9	2
IgA only	0	3
IgM only	0	7
IgG and IgA	6	0
IgG and IgM	2	4
IgG, IgA, and IgM	9	6
IgA and IgM	0	4

TABLE III. Subclass of IgG Antibodies Reactive With Heparin:PF4 Complexes

Group	N	IgG1 only	IgG2 only	IgG3 only	IgG1 and IgG3	IgG2 and IgG3
1	26	17	0	1	7	1
2	12	5	0	5	2	0

almost invariably have antibodies that recognize heparin:PF4 complexes appears to provide an important clue to the pathogenesis of this serious complication of anticoagulant therapy [6–10]. The thrombocytopenia characteristic of HITP is now thought to be mediated by immune complexes formed by the interaction of antibodies with heparin:PF4 complexes produced on or near the platelet surface. These complexes are thought to activate platelets by way of their membrane FcγRII receptors [7–9]. We have shown that the antibodies also recognize PF4 associated with heparin-like glycosaminoglycan molecules normally expressed on the surface of endothelial cells and have suggested that the thrombotic complications of HITP may, at least in part, be the result of this interaction [7]. Because the only Fc receptor known to be carried on platelets is specific for IgG Fc, only IgG antibodies should be able to mediate the binding of soluble immune complexes to platelets. IgM antibodies activate complement more efficiently than IgG or IgA antibodies and could theoretically cause platelet or endothelial cell activation or lysis by triggering the complement cascade. Therefore, the class and subclass of antibodies formed could influence symptomatology in patients sensitized by heparin.

The only previous reports in which the isotypes of antibodies reactive with heparin:PF4 complexes were determined are those of Amiral et al. [10,21] who per-

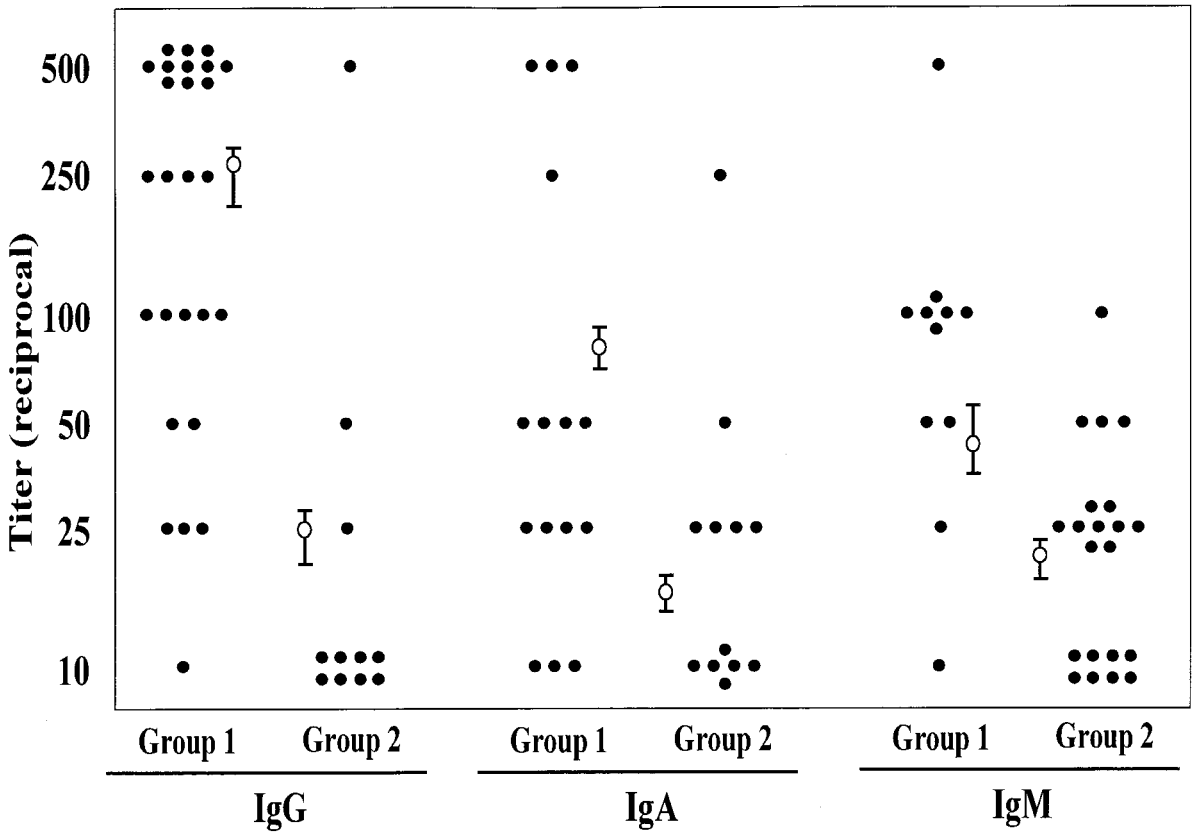

Fig. 2. Titers of IgG, IgA, and IgM antibodies reactive with heparin:PF4 complexes formed by patients in Group 1 (SRA positive with thrombocytopenia ± thrombosis) and Group 2 (SRA negative). Open circles and bars depict mean ± 1 SD. The differences between mean antibody titer in Group 1 vs. Group 2 were significant only for IgG ($P < 0.001$) (Mann-Whitney rank sum test).

formed isotyping for IgG, M, A, D, and E in two series of patients with Type II heparin-induced thrombocytopenia. In one series of 15 patients, 13 (87%) had IgG antibodies with or without IgA and/or IgM. Two had antibodies only of the IgM class. No antibodies of the IgD or IgE classes were identified [10]. In a second series of 38 patients, 26 (68%) had IgG antibodies together with IgM and/or IgA. The remaining 12 patients had antibodies of the IgM and/or IgA classes only [21]. Antibody titers were not reported, and IgG subclasses were not determined. Each of our 26 patients with Type II HITP had IgG antibodies accompanied by IgM and/or IgA. In contrast, the dominant antibody in the Group 2 patients, who formed antibody without developing thrombocytopenia, was IgM (Table II). The Group 2 patients had been exposed to unfractionated heparin (UFH) at cardiac catheterization and, subsequently, for 1–3 days after undergoing cardiac bypass surgery. It is likely, therefore, that the preponderance of IgM antibodies in this group reflects limited exposure to heparin and a primary antibody response to heparin:PF4. However, a patient recently described by Cadroy et al. [22], who had a history of Type II HITP, mounted a brisk IgM response when challenged with UFH again 3 years later. About half of the patients in both Groups 1 and 2 formed IgA antibodies, comparable to the 38% incidence observed by Amiral et al. in 16 patients [10]. The high frequency of IgA antibodies was unexpected and is currently unexplained.

The IgG antibodies formed by patients in both Groups 1 and 2 were almost exclusively of the IgG1 and IgG3 subclasses, as in most humoral immune responses. Only one antibody of the IgG2 class was identified in a Group 1 patient who also formed a strong IgG3 antibody. IgG2 Fc binds avidly to the His131 (nonresponder) allele of the platelet FcγRII receptor [15], which was found in two recent studies to be present more often than would be expected by chance in patients with HITP [13,14]. Our findings indicate that preferential formation of IgG2 antibodies does not provide an explanation for this relationship. These observations are at variance with a recent preliminary report, suggesting that antibodies of the IgG2 subclass are common in HITP [23]. It is unlikely that our failure to identify IgG2 antibodies was a consequence of selective sequestration of such antibodies during heparin administration because many of the patients studied did not develop thrombocytopenia and antibodies of the

IgG1 and IgG3 subclasses were readily detected in serial blood samples. Nor is it likely that IgG2 antibodies were selectively removed by treatment of plasma with Ecteola cellulose for removal of excess of heparin, since in separate experiments (data not shown) in which increasing amounts of heparin were added to HITP plasma samples, we found that Ecteola cellulose treatment had no effect on the titer of any class or subclass of immunoglobulin specific for PF4:heparin complexes.

Antibodies of the IgG class in patients experiencing severe thrombocytopenia with or without thrombosis were of much higher titer than those in patients without thrombocytopenia (Fig. 2). These findings are consistent with the possibility that the full-blown HITP syndrome is more likely to develop in patients who mount a high titer IgG response and who continue to receive heparin, although thrombocytopenia, with and without thrombosis has been described in patients whose antibodies were exclusively IgM and/or IgA [21]. Other influences, such as FcγRII receptor phenotype [13,14] or associated abnormalities of the regulation of hemostasis [24], also may be important in determining symptomatology.

Additional prospective studies are needed to determine whether continued exposure to heparin in asymptomatic patients with low titer antibodies will accentuate the humoral immune response, leading to more potent antibodies capable of causing thrombocytopenia and/or thrombosis. The description by Amiral and coworkers [10] of a single patient who was asymptomatic with antibody on Day 7 of heparin treatment, but who developed Type II HITP with thrombosis on Day 12, indicates that this sequence of events can take place in some patients.

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